Published in final edited form as:

J Chromatogr A. 2015 March 13; 1385: 28–34. doi:10.1016/j.chroma.2015.01.052.

Highly stabilized, polymer-lipid membranes prepared on silica microparticles as stationary phases for capillary chromatography

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Abstract

The ability to rapidly screen complex libraries of pharmacological modulators is paramount to modern drug discovery efforts. This task is particularly challenging for agents that interact with lipid bilayers or membrane proteins due to the limited chemical, physical, and temporal stability of conventional lipid-based chromatographic stationary phases. Here, we describe the preparation and characterization of a novel stationary phase material composed of highly stable, polymeric-phospholipid bilayers self-assembled onto silica microparticles. Polymer lipid membranes were prepared by photochemical or redox initiated polymerization of 1,2-bis[10-(2',4'-hexadecyloxy)decanoyl]-*sn*-glycero-2-phosphocholine (bis-SorbPC), a synthetic, polymerizable lipid. The resulting polymerized bis-SorbPC (poly(bis-SorbPC)) stationary phases exhibited enhanced stability compared to particles coated with 1,2-dioleoyl-*sn*-glycero-phosphocholine (unpolymerized) phospholipid bilayers when exposed to chemical (50mM triton X-100 or 50% acetonitrile) and physical (15 min sonication) insults after 30 days of storage. Further, poly(bis-SorbPC)-coated particles survived slurry packing into fused silica capillaries, compared to unpolymerized lipid membranes, where the lipid bilayer was destroyed during packing. Frontal chromatographic analyses of the lipophilic small molecules acetylsalicylic acid, benzoic acid, and salicylic acid showed > 44% increase in retention times ($P < 0.0001$) for all analytes on poly(bis-SorbPC)-functionalized stationary phase compared to bare silica microspheres, suggesting a lipophilic retention mechanism. Phospholipid membrane-functionalized stationary phases that withstand the chemical and physical rigors of capillary LC conditions can substantially increase

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6. Conflict of Interest

The authors declare no competing financial interest.

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the efficacy of lipid membrane affinity chromatography, and represents a key advance towards the development of robust membrane protein-functionalized chromatographic stationary phases.

1. Introduction

Cellular phospholipid membranes serve as barriers between the extracellular and intracellular environments, and provide a chemical environment for the constitution and dynamic function of transmembrane proteins that are key to regulating biological function. Many pharmaceutical modulators interact with the cell membrane either via direct partitioning into the membrane or through interactions with transmembrane proteins. Thus, drug screening assays often utilize intact cells to identify novel small molecule agonists and antagonists that act at the cellular membrane. Unfortunately, cell-based assays may suffer from irreproducibility due to biological variability within heterogeneous populations, and may be difficult to interpret due to the complexity of downstream signal transduction [1]. Affinity chromatography platforms that incorporate phospholipid membranes offer more controlled and robust alternatives to cell based assays for identifying compounds that interact with phospholipid membranes or with integrated membrane proteins [2]. Additionally, membrane-functionalized affinity platforms tend to minimize non-specific interactions [3].

Phospholipid membrane-functionalized affinity stationary phases have been utilized in chromatography to study partitioning and binding interactions. In immobilized liposome chromatography (ILC), liposomes are retained on a support matrix through steric, hydrophobic, covalent, or avidin-biotin interactions [4–7]. ILC has been primarily used to study small molecule partitioning through lipid membranes [8, 9] and interactions between peptides and phospholipids [10]. Membrane proteins have been immobilized in ILC stationary phases and used to study ligand binding [11–14]; however, the liposomes are formed by non-covalent interactions, which are inherently unstable. Thus, the stationary phases have limited lifetimes and reduced pressure stability, requiring low flow rates that reduce separation efficiency. Furthermore, ILC phases lack the chemical and mechanical stabilities needed to withstand common chromatographic solution conditions (e.g. organic solvents, varying ionic strength, etc.) or mechanical insults (air bubbles, shear forces, etc.), decreasing reproducibility and limiting their utility in complex separations [15–19].

Immobilized artificial membranes (IAMs) provide greater stability and reproducibility than ILCs for membrane-based chromatography. IAMs are prepared by covalently attaching lipids to a particle surface by their tail ends, resulting in a lipid monolayer-functionalized chromatographic stationary phase [20–22]. IAMs have been used to study interactions between small molecules and phospholipids [20, 23, 24]. Additionally, nicotinic acetylcholine receptors [25], μ and κ opioid receptors [26], and other membrane proteins [27] have been immobilized in IAMs and dissociation constants calculated against a series of small molecules by frontal chromatography. While these studies revealed similar trends to binding constants calculated using cell-based assays, there were quantitative differences which may have arisen from altered protein conformation due to interactions with the underlying silica support and the use of lipid monolayers rather than more biologically relevant bilayers [26, 28].

To maximize the efficacy of affinity chromatography for screening drug interactions at the phospholipid membrane, a more stable lipid environment that more accurately mimics the lipid bilayer found in cellular membranes is needed. Bilayer stability can be increased by incorporating cholesterol, adsorbing a protective overlayer, membrane tethering, or polymerizing phospholipid monomers [2]. Of these methods, directly polymerizing phospholipid monomers yields the most stable lipid bilayer structures.

Polymerizable phospholipids have been used in various analytical platforms to form stable phospholipid bilayers. Planar supported lipid bilayers (PSLBs) prepared by polymerizing 1,2-bis[10-(2',4'-hexadecyloxy)decanoyl]-*sn*-glycero-2-phosphocholine (bis-SorbPC) exhibit enhanced stability against surfactants, organics, and exposure to high vacuum [15–17]. Polymerized bis-SorbPC (poly(bis-SorbPC)) membranes have also been utilized in capillary zone electrophoresis as stable surface coatings for reducing the electroosmotic flow and minimizing non-specific adsorption of proteins [29, 30]. These polymerized surface coatings were stable to surfactant solutions, shear forces, applied electric fields, and dry storage [29, 31]. Additionally, rhodopsin, a transmembrane protein, was incorporated into PSLBs prepared from poly(bis-SorbPC) and retained its activity in the stabilized bilayer [19]. Together, these findings suggest that polymeric lipid bilayers may offer an excellent stationary phase environment for affinity chromatography studies of drug-membrane interactions. Though polymeric-lipid coatings have been used to minimize non-specific adsorption of proteins in a range of materials, to our knowledge, polymeric-lipid membranes have not yet been utilized as a stationary phase material in packed columns.

Here we describe the preparation of a poly(bis-SorbPC) bilayer-functionalized stationary phase material for membrane affinity chromatography. We assess the chemical, physical, and temporal stability of the polymerized-phospholipid bilayers and demonstrate their utility as a lipid-based stationary phase for frontal chromatography.

2. Experimental

2.1. Materials and reagents

1,2-bis[10-(2',4'-hexadecyloxy)decanoyl]-*sn*-glycero-2-phosphocholine (bis-SorbPC, $T_m = 29\text{ }^{\circ}\text{C}$) was synthesized according to previous protocols [32]. Before use, a 0.5 mL aliquot of 18 mg mL⁻¹ bis-SorbPC in 7:3 (v/v) methanol (MeOH): H₂O was purified on a C₁₈ column (Shimadzu Chromegabond WR C₁₈, 5 μm , 250 mm $\times$ 23 mm) using a 10 mL min⁻¹ gradient (Shimadzu BCM-20A controller and LC-8A pumps) of H₂O and MeOH. The MeOH volume in the mobile phase was increased from 50 – 70% in the first minute, increased from 70 – 85% over the next 20 min, increased from 85 – 100% over the next 40 min, and held at 100% for 5 min. The column was then flushed with 5% MeOH and 70% MeOH. All changes in the gradient were linear. The bis-SorbPC fraction was collected (elution time = 40 min), dried under vacuum, and washed 3 times with chloroform before dissolving in 500 μL chloroform. Purified bis-SorbPC was stored at $-80\text{ }^{\circ}\text{C}$. The concentration of bis-SorbPC was determined by UV absorbance at 258 nm ($\epsilon = 47100\text{ M}^{-1}\text{ cm}^{-1}$ in MeOH) (Model 440CCD Array UV-Vis Spectrophotometer; Spectral Instruments, Inc., Tucson, AZ). Sorbyl functional groups are light sensitive; thus, purification and preparation of bis-SorbPC-coated particles were performed under UV-free, yellow light.

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, $T_m = -17\text{ }^{\circ}\text{C}$) was purchased from Avanti Polar Lipids (Alabaster, AL). 3 μm and 5 μm silica particles (10% in water, size CV 10–15%) were purchased from Polysciences, Inc. (Warrington, PA). FM1-43 was purchased from Invitrogen (Eugene, OR). $\text{K}_2\text{S}_2\text{O}_8$, NaHSO_3 , and $(\text{NH}_4)_2\text{S}_2\text{O}_8$ were purchased from Sigma (St. Louis, MO). All other chemicals were purchased from Fisher (Pittsburgh, PA). H_2O was obtained from a Barnstead EasyPure UV/UF purification system. Buffers were filtered using membranes with 0.2 μm pores before use.

2.2. Preparation of lipid-coated particles

Chloroform from lipid stock solutions (DOPC and bis-SorbPC) was evaporated under Ar and lipids were dried by vacuum overnight (FreeZone 6, Labconco, Kansas City, MO). Lipid-coated particles were prepared by vesicle fusion. Briefly, dried lipids were suspended in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4), pH 7.4, to a concentration of 1 mg mL⁻¹. Lipid dispersions were sonicated at 40% output power until clarity using a cup horn sonicator (Model W-380, Heat Systems-Ultrasonics, Inc., Newtown, CT) to produce small unilamellar vesicles (SUVs) with a mean diameter (number distribution) of $43 \pm 15\text{ nm}$ (Malvern Zetasizer Nano-ZS, Worcestershire, United Kingdom). Non-porous silica particles (3.0 μm diameter) were added to the SUV dispersion at a surface area ratio of 1 silica: 6 lipid. The mixture was sonicated for 5 min and then allowed to rest for 30 min to promote vesicle fusion. Vesicle formation and fusion onto silica were performed at 35 $^{\circ}\text{C}$, above the phase transition temperature of both lipids. For some studies, silica particles (5 μm diameter) were used and the lipid deposition was performed at room temperature using a surface area ratio of 1 silica: 25 lipid.

Bis-SorbPC-coated particles were polymerized by one of three methods. Before all polymerizations, the lipid / silica dispersions were degassed by bubbling N_2 for 10 min. Lipid coatings on the 5 μm particles were polymerized using a UV pen lamp for 30 min. For 3 μm particles, the following conditions were utilized. UV-polymerized particles were irradiated with a 100 W Hg Arc lamp with an IR-water filter and a band pass filter (U-330, Edmund Optics, Barrington, NY) for 30 min while stirring.[33] Redox polymerizations were performed using two different methods. Bis-SorbPC-coated particles were polymerized in the presence of 65 mM $\text{K}_2\text{S}_2\text{O}_8$ and 22 mM NaHSO_3 for 3 h at 35 $^{\circ}\text{C}$ or at a mole ratio of 1 lipid: 300 $(\text{NH}_4)_2\text{S}_2\text{O}_8$: 300 NaHSO_3 for 1 h at 35 $^{\circ}\text{C}$. After preparation (DOPC-coated particles) or polymerization (bis-SorbPC-coated particles) each set of particles was washed by 6 cycles of centrifugation, removal of supernatant, and suspension in fresh PBS buffer.

2.3. Column packing with lipid-coated particles

Particles were packed in capillaries (50 μm or 75 μm i.d.; 360 μm o.d.; Polymicro Technologies, Phoenix, AZ) against Microtight filter assemblies (Upchurch Scientific, Oak Harbor, WA) [34–36]. The same filter assemblies were used as retaining frits at the column inlet. Particles were slurried in PBS buffer and packed at 1000 psi N_2 (50 μm) or at 560 psi He (75 μm) until an 8 cm packed bed was formed. After packing, the gas tank was turned off and the pressure was allowed to slowly decrease by bleeding out of the capillary over the next 1 h.

2.4. Evaluation of the stability of lipid coatings

Aliquots of particles were treated on days 1, 2, 15, and 30 with triton X-100 or acetonitrile (ACN) to monitor the stability of the lipid bilayer. Particles were aliquoted into individual tubes, centrifuged, and suspended in fresh PBS, pH 7.4, at 1.8×10^7 particles per 100 μL . Particles were treated with 25 μL of 50 mM triton X-100 at room temperature for 15 min or with 50% acetonitrile (v/v) and sonicated for 15 min. After incubation, particles were rinsed by 4 cycles of centrifugation, removal of supernatant, and suspension in fresh PBS to a final concentration of 1.8×10^8 particles mL^{-1} .

2.5. Fluorescence imaging of lipid-coated particles

FM1-43 was added to a particle slurry at a final concentration of 57 nM and allowed to intercalate into the lipid bilayer before imaging. Fluorescent images of particles were acquired on a Nikon Eclipse TE300 Quantum inverted microscope using a $40\times / 1.30$ N.A. oil objective. Fluorescent images were obtained using rhodamine filters: $\lambda_{\text{ex}} = 540 / 25$ nm; $\lambda_{\text{em}} = 620 / 60$ nm. Images were collected using a Quantix 57 back-illuminated CCD camera (Roper Scientific, Tucson, AZ) operated by MetaVue imaging software (Universal Imaging, Downingtown, PA). Images were analyzed using Image J [37]. Data is presented as the mean $\pm$ standard deviation (graphically represented as error bars) for $n = 3 \times 100$, with the intensity of 100 particles quantified for each of three batches of particles that were prepared separately.

Packed capillary columns were imaged with or without FM1-43, which was allowed to intercalate into the bilayers before excess was rinsed away. Images were acquired using the instrument described above, but with a $4\times / 0.13$ N.A. objective. Data is presented as the mean $\pm$ standard deviation for $n = 3 \times 3$, 3 measurements from 3 capillaries.

2.6. Flow cytometric analysis of lipid-coated particles

Flow cytometric analysis was performed using a FACScan flow cytometer (BDBiosciences, San Jose, CA) equipped with an air-cooled 15 mW Ar^+ laser tuned to 488 nm. The emission fluorescence of FM1-43 was detected and recorded through a $582 / 42$ nm bandpass filter in the FL2 channel. Data files consisting of approximately 50,000 events gated on forward scatter versus side scatter were acquired and analyzed using CellQuest PRO software (BD Biosciences). Appropriate electronic compensation was adjusted by acquiring particle populations with and without FM1-43. Data is presented as the average $\pm$ standard deviation for $n = 3 \times 50,000$, with 50,000 gated events quantified for each of three batches of particles that were coated and polymerized independently.

2.7. Frontal chromatography using poly(lipid)-coated particles

Frontal chromatography was performed using a lab-built instrument. Isocratic elution with PBS, pH 7.4, as the mobile phase utilized pressure driven flow that was applied by a Micropro Syringe Pumping System (Eldex, Napa, CA) connected to a Cheminert injection valve (Valco, Houston, TX) with a 600 nL sample loop and a dwell volume of 750 nL. The eluent was pumped at $1 \mu\text{L min}^{-1}$ at room temperature, resulting in 2750 ± 50 psi when a packed column was present in the instrument. The elution profile was monitored by ultraviolet absorbance detection (Model 500 Detector, ChromTech, Apple Valley, MN) at

220 nm. Signal from the detector was collected with an A/D converter (NI USB-6221, National Instruments, Austin, TX) and software written in LabVIEW (National Instruments). All samples were prepared at a concentration of 100 μ M in PBS buffer, pH 7.4. Statistical significance was determined using the two-tailed Student's t-test.

3. Results and Discussion

The development of poly(lipid) stationary phase matrices for liquid chromatography (LC) will enable high throughput discovery of pharmacological and physiological compounds that interact with lipid bilayers. A key long term motivation of this research is the preparation of highly stable, membrane protein-functionalized stationary phases for affinity chromatography. As a first step towards realizing this goal, we evaluated the physical and chemical stability of polymer-lipid bilayers prepared on silica particles that were packed into capillary LC columns and subsequently utilized for chromatographic analyses.

3.1. Chemical Stability of polymerized bis-SorbPC stationary phase

Fusing SUVs with a hydrophilic, silica surface results in rapid collapse of the vesicles to form a supported bilayer [38]. Supported lipid bilayers have been prepared on a range of substrates including planar silica substrates and curved micro and nanoparticle substrates. Previously, SUVs of bis-SorbPC were fused with planar silica substrates [15, 16], curved capillary surfaces [29, 30, 39], and ca. 100 nm silica nanoparticles [40] to form supported, polymerized phospholipid bilayers, suggesting that fusing bis-SorbPC SUVs with silica microparticles will yield a supported bilayer structure.

Figure 1 shows fluorescent images of silica and lipid-coated silica particles after staining with a lipid indicator, FM1-43. Previous studies with FM1-43 have illustrated high levels of fluorescence after intercalation of the dye into lipid membranes [29, 39, 41]. For comparison, both bis-SorbPC and DOPC, a natural lipid, were used to evaluate the stability of bilayer coatings. Natural phospholipid bilayers are unstable when exposed to common column preparation and/or separation conditions, including organic solvents, air bubbles, and shear forces [15–19]; thus, the stability of lipid bilayer coatings were examined first to ensure the bilayers would be compatible with separation systems. Silica (non-coated) particles, DOPC-coated particles, and poly(bis-SorbPC)-coated particles exhibited low fluorescence background prior to addition of FM1-43. After incubation with FM1-43, the fluorescence intensity of DOPC- and poly(bis-SorbPC)-coated particles increased, indicating the presence of lipid membranes on the surfaces of these particles. Additionally, the uniform fluorescence intensity indicates the presence of a bilayer membrane on the particle surface. To test the stability of the lipid bilayer coating, particles were exposed to surfactant (triton X-100) or a high concentration of organic solvent (50 % (v/v) acetonitrile, ACN), washed, and then stained with FM1-43. These conditions, while more harsh than would be expected for most analyses involving lipid stationary phases, were chosen to provide a clear indication of the stability enhancements obtained by utilizing poly(lipid) membranes. The fluorescence intensity of DOPC-coated particles decreased significantly after exposure to surfactant or organic solvents, indicating that the lipid bilayers were degraded. However, when poly(bis-SorbPC)-coated particles were exposed to either insult, the fluorescence

intensity was retained after staining with FM1-43, indicating the enhanced bilayer stability after polymerization of the lipids.

To increase the throughput of particle characterization, flow cytometry was used to study large groups of particles since the scatter associated with each particle could be correlated with the fluorescence. Table 1 shows the mean fluorescence intensity of silica, DOPC-coated, and poly(bis-SorbPC)-coated particles in the presence and absence of FM1-43 and after treatment with surfactant or organic solvent. This data correlates well with the images presented in Figure 1. Silica particles had low fluorescence under all conditions since lipids were not present on the particle surface. DOPC-coated particles exhibited increased fluorescence when stained with FM1-43 due to the presence of a lipid membrane; however, the bilayer coatings were unstable after exposure to surfactants or organic solvents as evidenced by the decreased fluorescence intensity following these conditions. When bis-SorbPC was polymerized using the UV or either redox initiation approach (with $K_2S_2O_8$ or $(NH_4)_2S_2O_8$ and $NaHSO_3$), the lipid coating resulted in fluorescence when stained with FM1-43. Additionally, the fluorescence was retained after exposure to surfactants or organic solvents, illustrating the enhanced stability of the bilayer coating after polymerization.

3.2. Temporal and physical stability of polymerized bis-SorbPC stationary phase

In addition to examining the stability of the bilayer coatings against common chemical insults, the long-term stability was analyzed by imaging. Particles were stained with FM1-43 prior to each experiment. Images were collected on the day of preparation, and again on days 15 and 30. Between experiments, particles were dispersed in PBS, pH 7.4, and stored at 4 °C. The data in Figure 2 shows that the poly(bis-SorbPC)-coated particles exhibited similar fluorescence intensities over the 30 day period, suggesting that the membranes were retained throughout this time frame. The polymerized bilayer coatings were also stable to surfactants and organic solvents over this duration, signifying that storage does not affect the stability of the bilayer coating. For the DOPC-coated particles, the fluorescence intensity was quantified only on the first day. Images of DOPC-coated particles collected on day 15 showed few fluorescent particles, indicating the bilayer coatings degraded during storage. The temporal stability of the poly(bis-SorbPC) membranes on silica particles was further supported by flow cytometry (Supplementary data, Figure S-1), where there was a consistent, but small decrease at day 30. The long-term stability of poly(bis-SorbPC) membrane coatings will allow for storage of chromatography columns without stationary phase degradation.

The physical stability of the bilayer coatings were analyzed by imaging capillaries packed with UV poly(bis-SorbPC)-coated silica particles, DOPC-coated silica particles, or bare silica particles in the presence or absence of FM1-43 (Figure 3). Only the capillaries packed with poly(bis-SorbPC)-coated particles showed high fluorescence intensities after exposure to FM1-43, indicating that the polymerized bilayers were stable to the shear forces required to pack capillary columns. Conversely, the capillaries packed with DOPC-coated particles exhibited diminished fluorescence, suggesting that DOPC lipid bilayers were removed by the shear forces generated during packing. Additional particle images (Supplementary data,

Figure S-2) illustrate that the poly(bis-SorbPC) coatings were stable when simultaneously exposed to high pressure (2900 psi) and organic solvents (25 – 40% ACN).

3.3. Improved polymerization conditions

The imaging and flow cytometry studies presented above show that poly(bis-SorbPC) membranes deposited on silica particles exhibit enhanced chemical, temporal, and physical stabilities compared to non-polymerized lipid bilayers (DOPC). However, aggregates of particles were observed after UV polymerization and these were exacerbated following membrane destabilizing conditions, e.g. surfactant or organic rinse (Figure 1). Interestingly, when bis-SorbPC was polymerized using either redox initiation, no aggregates were observed (Figure 1). Previous work by Ross et. al. showed that poly(bis-SorbPC) PSLBs that were prepared via UV or redox methods yielded different polymer structures [16]. In general, redox polymerization yielded a greater degree of crosslinking between the two leaflets of the bilayer and a longer polymer network (degree of polymerization, $X_n = 40 - 600$) compared to UV initiation ($X_n = 3 - 10$) [16]. Thus, when UV polymerized particles were exposed to surfactant or organic solvent, the lower crosslinking density of the polymer may be insufficient to prevent the loss of the outer leaflet of the bilayer, leading to aggregation of particles via the interaction of exposed lipid tails (see Supplementary data, Figure S-3 for a schematic representation).

A highly crosslinked polymer results when $K_2S_2O_8$ and $NaHSO_3$ are used as the redox couple to polymerize bis-SorbPC ($X_n = 40 - 600$) [15, 16]. The initiation reactions between $S_2O_8^{2-}$ and HSO_3^- are shown below [42].

Bisulfate (HSO_4^- , pK_a 1.9) is produced as a byproduct of initiation, resulting in an acidic solution. When bis-SorbPC lipids undergo redox-initiated polymerization, the decrease in pH does not affect the lipid structure or the resulting polymer; however, if future applications of this stationary phase material are to require membrane proteins incorporated into bis-SorbPC membranes prior to redox polymerization, the resulting decrease in pH may negatively affect protein conformation. Thus, we sought to identify conditions that would be more compatible with our long term goal of membrane protein-functionalized matrices. When $(NH_4)_2S_2O_8$ was substituted for $K_2S_2O_8$ in the redox reaction, a solution with approximately neutral pH resulted. The mixture could be buffered to pH 7.4 while still achieving polymerized membranes with no aggregation or other deleterious effects observed in stability studies (Figure 1 and Figure 2). Though the mechanistic differences between the redox reactions were not studied, redox polymerization using $(NH_4)_2S_2O_8$ should provide solution conditions that are more compatible for future incorporation of membrane proteins; thus, redox polymerizations using $(NH_4)_2S_2O_8$ and $NaHSO_3$ were employed for preparing poly(bis-SorbPC) coatings for frontal chromatographic analyses.

3.4. Frontal Chromatographic Analyses

To demonstrate the efficacy of poly(bis-SorbPC)-coated silica following packing into capillary columns, frontal analyses of three lipophilic small molecules that are known to cross cell membranes were performed using capillary LC. Figure 4 shows representative frontal chromatograms of acetylsalicylic (A), benzoic (B), and salicylic (C) acids using capillary columns packed with bare silica (black) or poly(bis-SorbPC)-coated silica (red) particles. The chemical structures for the analytes are shown in Supplementary data (Figure S-4). Retention times were defined as the maximum of the distribution in the first derivative plot (dashed lines) [43]. In each case, a clear increase in retention was observed when analytes were introduced to the poly(bis-SorbPC) stationary phase compared to silica stationary phase particles, suggesting lipophilic retention. Table 2 provides mean retention times and repeatability for the lipophilic analytes on silica and poly(bis-SorbPC)-coated stationary phases. Retention times increased with statistical significance ($P < 0.0001$) on the poly(bis-SorbPC) stationary phase relative to bare silica for each analyte.

Importantly, column performance was highly-repeatable. Frontal chromatograms yielded retention time relative standard deviations less than 4% for acetylsalicylic acid and less than 1% for benzoic acid and salicylic acid ($n = 4$) over the course of 1 week, values that are on par with silica particles lacking a coating. This is particularly noteworthy when considering the operating pressure for each run exceeded 2700 psi. Thus, not only do poly(bis-SorbPC)-coated particles withstand slurry packing, but also high pressures associated with the separation, which would delaminate unpolymerized lipid coatings and lipid architectures. These findings extend the application of lipid membrane affinity chromatography to conditions amenable to high efficiency separations which we expect to enable screening of highly complex drug-candidate mixtures for their lipid membrane interactions.

4. Conclusions

Poly(bis-SorbPC) coatings on silica particles exhibited increased chemical, temporal, and physical stability compared to unpolymerized phospholipid bilayer coatings (DOPC). Redox-initiated polymerization yielded bilayers with greater stability than UV poly(bis-SorbPC) coatings, which aggregated after exposure to chemical insults. Additionally, chromatographic frontal analyses of small molecule analytes showed repeatable lipophilic retention on poly(bis-SorbPC)-coated particles polymerized with a neutral redox polymerization mixture, $(\text{NH}_4)_2\text{S}_2\text{O}_8 / \text{NaHSO}_3$, indicating that the highly stabilized lipid membrane environment of the poly(lipid) stationary phase presents an excellent platform for further development of biomimetic stationary phases. The current platform will enable high efficiency analyses of lipophilic drugs to determine partition coefficients as these molecules interact with phospholipid bilayers. Future work in developing this stationary phase will target improved stationary phase capacity by utilizing porous silica particles, and incorporating membrane proteins for receptor affinity chromatography.

Poly(bis-SorbPC) stationary phases show enhanced stability and high repeatability compared to ILCs. The presence of a full phospholipid bilayer may also make poly(lipid) stationary phases a more biologically relevant mimic of the cellular membrane than the lipid monolayers found in IAMs. We expect these key differences from established lipid

membrane stationary phase materials will present an excellent opportunity for new affinity chromatography approaches to study physiological and pharmaceutical modulators of phospholipid membranes and/or membrane proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded in part by the National Institutes of Health (GM095763) and National Science Foundation (CHE-0518702). ESG was supported by a graduate training fellowship through the Biological Chemistry Program (NIH T32 GM008804). Flow cytometry data was collected in a core facility funded in part by the National Cancer Institute (NIC CCSG CA023074).

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Highlights

- A polymerized phospholipid bilayer-based chromatographic stationary phase is characterized and demonstrated.
- The stationary phase survives chemical and physical insults inherent in high pressure capillary liquid chromatography, with a stable shelf life of at least 30 days.
- Frontal chromatography shows lipophilic retention of acetylsalicylic, benzoic, and salicylic acids.

Figure 1.

Representative fluorescence images of silica particles, DOPC-coated particles, and poly(bis-SorbPC)-coated particles, with UV, $K_2S_2O_8 / NaHSO_3$, and $(NH_4)_2S_2O_8 / NaHSO_3$ referring to the bis-SorbPC polymerization method. After incubation with FM1-43, DOPC-coated and poly(bis-SorbPC)-coated particles exhibit increased fluorescence, indicating the presence of lipid bilayers on the surface of the particles. After exposing the lipid-coated particles to surfactant (triton X-100) or organic solvent (ACN), washing, and staining with FM1-43, only the poly(bis-SorbPC)-coated particles exhibit fluorescence, illustrating that the poly(bis-SorbPC) coatings are maintained after exposure to these insults and have greater chemical stability than unpolymerized DOPC coatings.

Figure 2.

Long-term stability of lipid bilayer coatings on silica particles measured by fluorescence microscopy using FM1-43 as a membrane stain. UV, $K_2S_2O_8 / NaHSO_3$, and $(NH_4)_2S_2O_8 / NaHSO_3$ refer to the method used for polymerization of bis-SorbPC. Particles were exposed to surfactant (triton X-100) or organic solvent (ACN), washed, and stained with FM1-43 before imaging. For all poly(bis-SorbPC)-coated particles the maintained fluorescence after exposure to chemical insults over a 30 day period illustrates that the polymerized-bilayer coatings are temporally stable. Particles were stored dispersed in PBS at 4 °C between timepoints.

Figure 3.

Fluorescent images of capillaries (75 μm i.d., 360 μm o.d.) packed with UV poly(bis-SorbPC)-coated particles (A, B), bare silica particles (C, D), and DOPC-coated particles (E, F). Capillaries were imaged with (A, C, E) or without (B, D, F) FM1-43. (G) Mean fluorescence intensity data for columns A – F. The fluorescent intensities illustrate that the poly(bis-SorbPC)-bilayer coatings are retained on the particles after packing, whereas the DOPC bilayers are degraded, likely due to the shear forces associated with packing columns.

Figure 4.

Chromatographic frontal analyses of lipophilic small molecules. Frontal analyses of acetylsalicylic acid (A), benzoic acid (B), and salicylic acid (C) show increased retention on poly (bis-SorbPC) stationary phases (red) when compared to bare silica stationary phases (black). Retention times were determined from first derivative plots (dashed lines).

Table 1

Chemical stability of lipid bilayer coatings analyzed by flow cytometry

Silica Particle Coating	Particle Fluorescence			
	No Treatment	+ FM1-43	+ Triton X-100 + FM1-43	+ 50% ACN + FM1-43
None	3 ± 3	4 ± 4	8 ± 8	5 ± 4
DOPC	3 ± 1	670 ± 190	23 ± 19	46 ± 54
UV poly(bis-SorbPC)	4 ± 2	890 ± 100	630 ± 140	730 ± 100
K ₂ S ₂ O ₈ / NaHSO ₃ poly(bis-SorbPC)	3 ± 1	1000 ± 160	1100 ± 280	1200 ± 230
(NH ₄) ₂ S ₂ O ₈ / NaHSO ₃ poly(bis-SorbPC)	3 ± 1	1300 ± 290	1400 ± 230	1400 ± 300

Table 2

Frontal chromatography retention analyses

<i>Analyte</i>	Silica		poly(bis-SorbPC)	
	<i>Retention time (min)</i>	<i>RSD (%)</i>	<i>Retention time (min)</i>	<i>RSD (%)</i>
Acetylsalicylic acid	10.1 ± 0.2	1.7	14.6 ± 0.6	3.8
Benzoic acid	9.6 ± 0.2	1.9	14.1 ± 0.1	0.9
Salicylic acid	9.7 ± 0.1	1.0	14.3 ± 0.1	0.4